Polish Journal of Environmental Studies Vol. 8, No. 4 (1999), 215-220

Original Research

Chitinase Activity Production by Planktonic, Benthic and Epiphytic Bacteria Inhabiting the Moty Bay of the Jeziorak Lake (Poland)

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Received 10 April, 1999
Accepted 20 April, 1999

Abstract

A study on the occurrence and chitinolytic activity of planktonic, benthic and epiphytic bacteria was carried out in the Moty Bay of Lake Jeziorak near Ilawa. The greatest number of chitinolytic bacteria occurred on macrophytes. In terms of percentages, the chitinolytic bacteria share among heterotrophic bacteria was much greater in epiphytic and planktonic bacteria than in benthic ones. When confronted with colloidal chitin, all the investigated strains showed maximum chitinolytic activity after 96 h incubation and the minimum after 48 hours. The greatest total chitinase activity was recorded in planktonic bacteria, while specific activity - in epiphytic bacteria.

Keywords: chitinolytic bacteria, heterotrophic bacteria, bottom sediments, surface water, macrophytes, chitinases

Introduction

Chitin is a homopolysaccharide, a linear polymer made of monomers of N-acetyloglucosamine connected by glycoside β- (1-4) bonds. It is a supporting substance in an outer skeleton of low-organized animals and in fungi cell walls. It is very common in the water of water bodies and in bottom sediments. Its main suppliers are Crustacea, the outer skeletons of which are made of chitin [15, 10]. Chitin is also present in diatoms, protozoa, insects, arachnids and nematodes [18]. The marine environment has the greatest resources of chitin. Most of it is produced as zooplankton carapaces [9]. Phytoplankton is also a major chitin source. It has been estimated that 10^{10} tons of assimilated carbon is annually included in it [16, 17].

The biologic degradation of chitin is accompanied by specific enzymes: chitinase and chitobiase. The end product of chitin decomposition is N-acetyloglucosamine, which is very likely to provide many organisms with carbon and nitrogen [8]. Chitinolytic enzymes are produced by many bacteria and fungi [12], and also by plants and invertebrates [18]. According to Rheinheimer [15], the most active bacteria are those of *Pseudomonas* and *Vibrio* genera and also actinomycetes of the *Micronomospora* genus.

Material and Methods

Site of the Study

The study was carried out in Lake Jeziorak's Moty Bay. Lake Jeziorak is located in the Hawskie Lake District and makes part of the Vistula-Drweca catchment area. It is a post-glacial lake of a meridian-like placement. The lake surface is 32.3 km^2, length is 27.5 km, mean width is about 1.2 km, and maximum depth is 12.0 m; the mean depth is about 5.7 m. The lake is one of eutrophic water bodies [7]. The shoreline is well developed and covers three bays, including Moty Bay. The lake's banks are grown with mixed forest. It is an area of shallow water, averaging 2.5 m deep, the bottom sediments of which are very thick (about 8 m) and contain much organic debris lying loosely on the bottom.

Sampling

The study material contained bacteria isolated from water and bottom sediments of the littoral, sublittoral and pelagial zones and also from the bay's macrophytes surfaces. The water was sampled from the depth of 10-20 cm with a sterile pipette by means of a pipette automatic pump (Pipet-boy, DeVille), and then poured into sterile bottles sealed with rubber stoppers. The bottom sediments were sampled by means of a self-constructed tube scoop and their surface layer (down to 5 cm) was aseptically transferred into sterile, top-twisted jars. Also, a 15 cm long common reed *(Phragmites australis (Cav.)Trin. ex Steudel),* lesser reedmace *(Typha angustifolia L.)* from subsurface water, and yellow waterlily *(Nuphar lutea L.)* were collected for examination and were placed in sterile glass jars. All samples were put into thermoisolated containers with ice (temperature inside did not exceed ±7°C) and then transferred into a laboratory, where they were immediately analyzed. The study material was collected between June and November 1997.

Heterotrophic Bacteria Number

The number of heterotrophic bacteria (CFU) in the samples of water, bottom sediments and macrophytes were determined by means of the spread plates method, inoculating the material from respective solutions in three parallel repetitions in an iron-peptone agar medium, according to Ferrer, Stapert, and Sokolski [5]. In order to estimate the heterotrophic bacteria number in the bottom sediments and those inhabiting plant surfaces, 10.0 g of sediments or of fresh plant mass were sampled and poured over by 90 cm^3 of sterile buffer water [3]. All this was ground for 2 min. with the use of a Devimix laboratory homogenizer. Following that, the homogenizates were diluted with sterile buffer water and inoculated onto the medium surface. All inoculations were incubated at 20°C for 7 days and then the newly grown bacteria colonies were counted, converting the result into 1 cm^3 water or 1 g dry weight of the sediments/ dry weight plant.

Chitinolytic Bacteria Number

The number of chitinolytic bacteria in the examined samples were determined with the use of the spread plates method applying the medium consisting of the following: peptone (peptobak) - 1.0 g, iron sulphate - 0.1 g, ammonia sulphate - 0.1 g, iron gluconate - 0.1 g, yeast extract - 0.1 g, colloidal chitin - 7.0 g of dry mass, agar - 15.0 g, tap water - 1.0 dm^3, pH 7.2 -7.4. The colloidal chitin had been prepared according to Lingappa and Lock wood [11]. The plates with inoculations were incubated at 20°C for 14 days. The bacteria colonies which were accompanied by clearing zones were regarded as chitinolytic bacteria. Those strains were isolated and inoculated into semi-liquid medium as above (5.0 g of agar/ dm^3). After 7 days of incubation at 20°C the culture purity was inspected staining the specimens with the use of the Gram method. The strains were stored in a fridge at 4°C for further study; they were transferred into a fresh semi-liquid medium every 2 months.

Examination of the Bacteria Chitinoclastic Activity

Strains isolated from water (30), from bottom sediments (20) and from macrophytes (25) were used for this study. All those strains initially revealed a great chitinolytic activity by forming 5-10 mm zones of chitin hydrolysis around colonies. Selected strains were multiplied on slants with iron-peptone agar medium for 72 hours at 20°C. Then they were washed off with 2.5 cm^3 of sterile buffer water

Table 1. Total number of heterotrophic and chitinolytic bacteria inhabiting the Moty Bay of Lake Jeziorak.

Date of sampling	surface water •			bottom deposit ••			macrophytes ••		
	littoral	sublittoral	pelagial	littoral	sublittoral	pelagial	yellow waterlily	common reed	lesser reedmace
18.06.1997	* 26.0	5.7	3.4	6635.0	2437.0	2000.0	10730.4	11334.7	10412.6
	** 7.0	0.87	0.56	1134.6	204.7	160.0	4266.3	3229.3	1248.9
22.07.1997	* 2.8	1.6	0.9	5902.0	4935.0	1302.0	26220.9	46306.5	26647.9
	** 0.9	0.5	0.2	861.7	616.9	109.4	4198.9	5299.2	5200.5
21.08.1997	* 4.8	3.0	2.8	12507.0	10000.0	9000.0	7983.2	8430.8	34945.2
	** 1.5	0.9	0.5	787.9	520.0	414.0	367.6	928.3	1032.9
24.09.1997	* 6.9	2.9	1.1	2000.0	1703.0	1506.0	21905.4	6470.3	19331.9
	** 0.83	0.15	0.0	172.0	71.5	0.0	4857.9	1735.9	7062.6
21.10.1997	* 5.0	2.1	1.0	870.0	440.0	300.0	1932.4	8259.7	8203.8
	** 0.24	0.042	0.0	21.7	0.0	0.0	497.2	1019.7	519.7
21.11.1997	* 4.8	2.0	0.9	230.0	200.0	100.0	nb	nb	nb
	** 0.0	0.0	0.0	0.0	0.0	0.0			
average	* 8.4	2.9	1.7	4690.7	3285.8	2368.0	13574.5	16160.4	19908.3
	** 1.75	0.4	0.2	496.3	235.5	113.9	2837.6	2442.5	3012.9

Explanations: * – number of heterotrophic bacteria (CFU), ** – number of chitinolytic bacteria (CFU), • – number of bacteria x $10^3/cm^3$, •• – number of bacteria x 10^3/g dry weight plant or g dry weight bottoms, nb – non studies.

[3]. The thus acquired 0.5 cm^3 suspension was used to inoculate 15 cm^3 doses of liquid iron-peptone medium with pH 7.0. The bacteria were incubated at 20°C until the phase of their logarithmic development was captured and the optic density of the culture was marked on the "Marcel 330 Pro" spectrophotometer a wavelength 565 nm. The bacteria rinsed in sterile buffer water made a suspension of absorbancy E = 0.3 (which corresponds to 10^9 bacterial cells per 1 cm^3), and about 0.5 cm^3 of it was introduced into 100 cm^3 Erlenmayer bulbs containing 20 cm^3 of liquid medium composed of the following: colloidal chitin - 2%, peptone (peptobak) - 1.0 g, iron gluconate - 0.1 g, ammonia sulphate - 0.1 g, iron sulphate - 0.1 g, yeast extract - O.lg, tap water - 1.0 dm^3, pH - 7.2-7.4. Chitinase (crude enzyme) production and activity were estimated after 2, 4, 6, 8, 10, and 12 days of incubation on the basis of chitin N-acetyloglucosamine according to Reissig, Strominger and Leloir [14] which was released during hydrolysis. Chitinase activity (U) was determined on the basis of the number of mmoles of released N-acetyloglucosamine converted into: 1 cm^3 of after-culture liquid, per hour - total activity, 1 mg of protein in after-culture liquid, per hour - specific activity.

Protein content was determined with the Bradford [2] method.

Chitinolytic Bacteria Identification

The chitinolytic bacteria identification was done according to the pattern for freshwater bacteria suggested by Allen, Austin and Colwell [1].

Results

The number of chitinolytic bacteria inhabiting surface water, bottom sediments and macrophytes in the Moty Bay of Lake Jeziorak is presented in Table 1. Data reveal that the majority of chitinolytic bacteria occur on macrophytes surfaces; most of them were recorded on the lesser reedmace (3012.9 x 10^3 cell/g of dry weight on the average). The chitinolytic bacteria number changed in surface water and bottom sediments. Most of them were recorded in the littoral, significantly less in sublittoral and the minimum in the pelagial zone.

Chitinolytic bacteria occurred most frequently on macrophytes in September on lesser reedmace (7062.6 x 10^3 cell/g of dry weight plant). On the other hand, in water and bottom sediments most of them were noted in June in littoral (7.0 x 10^3 cell/cm^3 and 1134.6 x 10^3 cell/g of dry weight sediment). The smallest amounts occurring in water were recorded in October in sublittoral (0.042 x 10^3 cell/cm^3), and in bottom sediments in littoral (21.0 x 10^3 cell/g of dry weight sediment). In November, no chitinolytic bacteria were found at all.

Table 2 presents the bacteria percent share in chitin decomposition. As it shows, chitinolytic bacteria were most abundant among epiphytes, and the greatest number of them were recorded on yellow waterlily (21.6 % on the average). The smallest amounts of chitinoclasts were found in bottom sediments in the pelagial zone (3.5 % on the average). The data included in Table 2 also reveal that chitinolytic bacteria amounted to 0.0%-32.1% in surface water, with their maximum in littoral in July. In bottom sediments they made between 0.0% and 17.1 % with their maximum in littoral in June. Chitinoclasts made between 2.9% and 39.75 on macrophytes and the majority was found on yellow waterlily in June.

The study on chitinolytic bacteria species composition (Tab. 3) revealed that most frequent were the following species: *Bacillus cereus, Bacillus pumilus, Bacillus firmus, Aeromonas sp., Vibrio fluvialis, Enterobacter aerogenes.* Among planktonic bacteria the following were the most frequently occurring species: *Bacillus cereus* and *Bacillus pumilus* (16.4%, 14.9% respectively). Among epiphytes it was *Bacillus pumilus* (25.0%) and *Enterobacter aerogenes* (21.0%), and among the benthic bacteria *Aeromonas sp.* (26.3 %).

The study on chitinoclastic bacteria activity (Figs. 1-2) revealed that the total and specific activity was lowest among epiphytic bacteria. The greatest total activity was recorded among planktonic bacteria (0.0070 U/cm^3 average on one strain). Among the investigated strains there was noted a change in chitinase activity with the incubation time. After 96 hours of incubation all the examined strains revealed maximum activity, which then declined. Table 4 presents chitinoclastic activity of selected planktonic, benthic and epiphytic bacteria. It revealed that the greatest total and specific activity was shown by the strains of *Alcaligenes denitryficans* (0.012 U/cm^3, 0.042 U/mg), *Flavo-*

Table 2. Percent share of planktonic, benthic and epiphytic bacteria capable of chitine decomposition.

Habitat		Date of sampling						
		18.06.1997	22.07.1997	21.08.1997	24.09.1997	21.10.1997	21.11.1997	average
surface water	littoral	26.9	32.1	31.3	12.0	4.8	0.0	18.5
	sublittoral	15.3	31.3	30.0	5.2	2.0	0.0	13.9
	pelagial	16.5	23.0	18.6	0.0	0.0	0.0	9.7
bottom deposit	littoral	17.1	14.6	6.3	8.6	2.5	0.0	8.2
	sublittoral	8.3	12.5	5.2	4.2	0.0	0.0	5.0
	pelagial	8.0	8.4	4.6	0.0	0.0	0.0	3.5
macrophytes	yellow waterlily	39.7	16.0	4.6	22.2	25.7	nb	21.6
	common reed	28.5	11.4	11.0	26.8	12.3	nb	18.0
	lesser reedmace	12.0	19.5	2.9	36.5	6.3	nb	15.4

Explanation: nb – non studies.

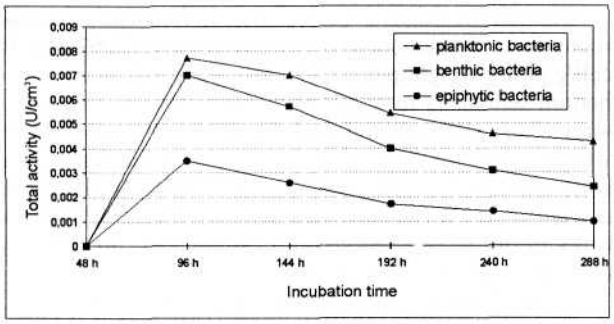

Fig. 1. Total activity of chitinases production by planktonic, benthie and epiphytic bacteria isolated from Moty Bay of Lake Jeziorak (average).

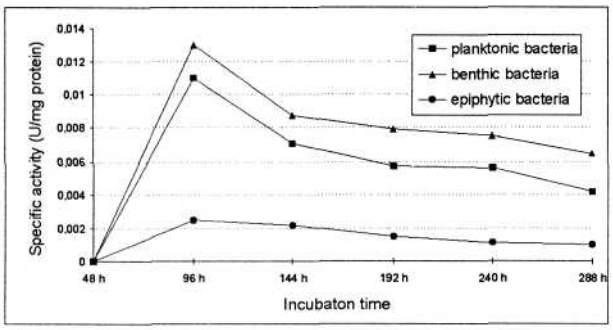

Fig. 2. Specific activity of chitinase production by planktonic, benthic and epiphytic bacteria isolated from Moty Bay of Lake Jeziorak (average).

bacterium sp. (0.075 U/cm^3, 0.15 U/mg) which were isolated from water; *Aeromonas sp.* (0.014 U/cm^3, 0.011 U/mg) which was isolated from bottom sediments, and *Arthrobacter sp.* (0.010 U/cm^3, 0.0060 U/mg) which usually inhabits macrophytes.

Discussion

Chitin is the most frequently occurring structural element of many invertebrates and fungi. Its main resources in water bodies are outer skeletons of insects and Crustacea. Quite possibly chitin degradation is accompanied by enzymes-chitinases, which attack the polymer in many sites which, consequently, results in the occurrence of slight amounts of N-acetyloglucosamine and chitobiosis and chitotriosis in the substrate [17]. Chitinases are normally produced by a large number of organisms. One of the main parts is played by the bacteria among them, which in the process of chitin decomposition release N-acetyloglucosamine into the environment, which makes an important source of carbon and nitrogen for themselves and for many other organisms. In the West Baltic and its fiords from 5 to some thousands chitin decomposing bacteria were found in 1 cm^3. According to Rheinheimer [15] the importance of chitin psychrophylic destruents is great as they produce large amounts of chitin in the marine environment, e.g. copepoda.

This paper's results indicate that the chitinolytic bacteria number in Lake leziorak's Moty Bay was greatest on macrophytes. It has been suggested that it is associated with the occurrence of many different organisms, e.g. insects which mine and inhabit plant surfaces. The bacteria horizontal number decline in surface water and in the bottom sediments (the greatest number of them was recorded in littoral, significantly fewer in sublittoral and the minimum in pelagial) may be associated with a better availability and abundance of chitin sources in littoral than in sublittoral or pelagial. It was recorded that the chitinolytic bacteria number was the greatest in June and July. It may be assumed that such development and activity of these bacteria were affected by an abundance of organisms and favourable environmental conditions, such as optimal temperature.

The study on chitinolytic bacteria species composition revealed that the most frequently occurring were the following species: *Bacillus cereus, Bacillus pumilus, Bacillus firmus, Aeromonas sp., Vibrio fluvialis, Enterobacter aerogenes, Flavobacterium sp., Alcaligenes denitrificans.* These data partly confirmed those obtained by Donderski [4], who also recorded the presence of some other chitin decomposing bacteria of the *Achromobacter, Pseudomonas, Chromobacterium* and *Nocardia* genera. Among 50 bacteria isolated from the soil Schlegel [17] found chitinolytic abilities in bacteria of the *Flavobacterium* group, *Bacillus, Cytophaga* and *Pseudomonas* genera. Rheinheimer [15] detected the species of *Pseudomonas cryothasia* and *Vibrio algosus* in the Baltic and Northern Seas. Those bacteria occurred abundantly on the carapaces of dead cancers.

Laboratory analyses revealed low values of the activity of chitinases produced by the bacteria under investigation.

Table 3. Species composition of chitinolytic bacteria (share in %).

Species name	Habitat		
	surface water	bottom deposit	macrophytes
Bacillus cereus	16.4	5.3	0.0
Bacillus pumilus	14.9	5.3	25.0
Bacillus megaterium	4.5	7.8	0.0
Bacillus firmus	10.5	13.2	4.2
Aeromonas hydrophila	4.5	13.2	0.0
Aeromonas sp.	10.4	26.3	16.7
Flavobacterium sp.	5.9	2.6	4.0
Acinetobacter sp.	4.5	2.6	4.2
Flexibacter cytophaga	1.5	0.0	0.0
Micrococcus varians	3.0	0.0	0.0
Enterobacter aerogenes	1.5	2.6	21.0
Serratia sp.	7.5	5.3	4.0
Cytophaga hutchinsonii	0.0	2.6	0.0
Vibrio fluvialis	4.5	13.2	8.3
Alcaligenes denitrificans	5.9	0.0	0.0
Alcaligenes sp.	1.5	0.0	4.2
Arthrobacter sp.	3.0	0.0	8.4

Table 4. Chitinoclastic activity of some planktonic, benthic and epiphytic bacteria isolated from Moty Bay of Lake Jeziorak.

Name of bacteria	Habitat	Incubation time					
		48 h	96 h	144 h	192 h	240 h	288 h
Bacillus pumilus	surface water	* 0.0 ** 0.0	0.0092 0.016	0.0082 0.0048	0.0054 0.0048	0.0027 0.0021	0.0031 0.0014
Aeromonas hydrophila		* 0.0 ** 0.0	0.0046 0.0036	0.0078 0.0030	0.0050 0.0029	0.0032 0.0023	0.0027 0.0023
Alcaligenes denitrificans		* 0.0 ** 0.0	0.012 0.042	0.0057 0.015	0.0036 0.0061	0.0033 0.0061	0.0024 0.0061
Flavobacterium sp.		* 0.0 ** 0.0	0.075 0.15	0.057 0.044	0.043 0.031	0.030 0.030	0.017 0.019
Bacillus cereus		* 0.0 ** 0.0	0.0044 0.0022	0.0014 0.0015	0.0014 0.0080	0.0012 0.0023	0.0012 0.0013
Vibrio fluvialis		* 0.0 ** 0.0	0.0088 0.0042	0.0069 0.0036	0.0053 0.0028	0.0039 0.0016	0.0036 0.0013
Aeromonas sp.	bottom deposit	* 0.0 ** 0.0	0.014 0.011	0.012 0.011	0.010 0.010	0.0065 0.010	0.006 0.0010
Vibrio fluvialis		* 0.0 ** 0.0	0.0021 0.0029	0.0020 0.0025	0.0018 0.0024	0.0015 0.0021	0.0010 0.0018
Serratia sp.		* 0.0 ** 0.0	0.0077 0.0070	0.0022 0.0020	0.0016 0.0020	0.0011 0.0015	0.0010 0.0012
Bacillus pumilus		* 0.0 ** 0.0	0.0015 0.0036	0.0013 0.0031	0.0012 0.0030	0.0010 0.0030	0.0010 0.0027
Cytophaga hutchinsonii		* 0.0 ** 0.0	0.0038 0.0025	0.0035 0.0024	0.0030 0.0024	0.0027 0.0018	0.0010 0.0012
Bacillus pumilus	macrophytes	* 0.0 ** 0.0	0.0023 0.0062	0.0032 0.0011	0.0015 0.00088	0.0013 0.00071	0.0010 0.00035
Aeromonas sp.		* 0.0 ** 0.0	0.0077 0.0010	0.0070 0.0011	0.0070 0.0010	0.0080 0.0010	0.0069 0.0010
Enterobacter aerogenes		* 0.0 ** 0.0	0.0023 0.0017	0.0015 1.0013	0.0014 0.0012	0.0013 0.001	0.0011 0.00052
Arthrobacter sp.		* 0.0 ** 0.0	0.010 0.0060	0.0082 0.0032	0.0017 0.0015	0.0016 0.0015	0.0010 0.0013

Explanations: * – total activity of chitinases (U/cm^3), ** – specific activity of chitinases (U/mg protein)

Most probably it was associated with the presence of chitotrioses and chitobioses in the after-culture liquid and slight amounts of N-acetyloglucosamine, which might hamper chitinases production by bacteria, thus making it undetectable. Besides, some bacteria might use the end product of chitin decomposition as a source of carbon and nitrogen. It should also be taken into account that the study was done on impure enzymes, the inhibitors of which might have been present in the after culture liquid. Epiphytic bacteria-produced chitinase activity has always been lower than with planktonic and benthic bacteria. Our data also state that chitinase activity produced by the bacteria under investigation underwent changes with incubation times. Maximum activity was recorded after 96 hours; then activity declined. On one hand it may be presumed that once the colloidal chitin had decomposed, the bacteria started to use its decomposition product as an additional food source. On the other hand, however, it is quite probable that N-acetyloglucosamine forms a catabolic inhibitor of the chitinases synthesis, as happens in the case of glucose in relation to celulases synthesis. While analyzing chitinolytic enzyme production in *Aeromonas sp.*, Huang, Chen and Su [10] found the highest activity in chitinases after 50 hours of incubation. On the other hand, the highest activity in *Bacillus pabuli Kl* was recorded after 120 hours of incubation, followed by its decline (Frandberg and Schniirer [6], which may also depend on the bacteria development phases.

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